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Immortal coils: Conserved dimerization motifs of the *Drosophila* ovulation prohormone ovulin

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Abstract

Dimerization is an important feature of the function of some proteins, including prohormones. For proteins whose amino acid sequences evolve rapidly, it is unclear how such structural characteristics are retained biochemically. Here we address this question by focusing on ovulin, a prohormone that induces ovulation in *D. melanogaster* females after mating. Ovulin is known to dimerize, and is one of the most rapidly evolving proteins encoded by the *Drosophila* genome. We show that residues within a previously hypothesized conserved dimerization domain (a coiled-coil) and a newly identified conserved dimerization domain (YxxxY) within ovulin are necessary for the formation of ovulin dimers. Moreover, dimerization is conserved in ovulin proteins from non-*melanogaster* species of *Drosophila* despite up to 80% divergence. We show that heterospecific dimers can be formed in interspecies hybrid animals and in two-hybrid assays between ovulin proteins that are 15% diverged, indicating conservation of tertiary structure amidst a background of rapid sequence evolution. Our results suggest that because ovulin's self-interaction requires only small conserved domains, the rest of the molecule can be relatively tolerant to mutations. Consistent with this view, in comparisons of 8510 proteins across 6 species of *Drosophila* we find that rates of amino acid divergence are higher for proteins with coiled-coil interaction domains than for non-coiled-coil proteins.

Keywords

seminal proteins; reproduction; *Drosophila*; evolution; dimerization; coiled-coil; ovulin prohormone

1. Introduction

Insect seminal fluid makes important contributions both to fertilization and to changes in the mated female's physiology and behavior. In *Drosophila* and many other insects, products of the male accessory glands are key contributors to the seminal fluid (for reviews, see Chapman and Davies 2004, Gillott 2003, Ravi Ram and Wolfner 2007a). The functions of the accessory gland products are best understood in *D. melanogaster*, although recent studies have begun to characterize the seminal fluid of a number of other insects (e.g., Almeida and DeSalle 2009, Andres et al. 2006, Baer et al. 2009, Braswell et al. 2006, Collins et al. 2006, Davies and Chapman 2006, Dottorini et al. 2007, Sirot et al. 2008, Wagstaff and Begun 2005, Weiss and Kaufman 2004). In *D. melanogaster*, mated females actively reject copulation attempts by

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subsequent males, increase their rates of egg production, ovulation, egg-laying, and feeding, and store sperm for up to two weeks. In the context of multiple mating (a common event in many species of *Drosophila*, including *D. melanogaster* - Frentiu and Chenoweth 2008, Harshman and Clark 1998), sperm storage in turn sets the stage for sperm competition, whereby sperm from different males compete for fertilization opportunities. Moreover, mated *D. melanogaster* females suffer a 'cost of mating', in that their lifespan is reduced in comparison to virgins. Products of the male's accessory gland are necessary for all of these post-mating responses: Males lacking accessory glands, or whose accessory gland secretory cells have been largely ablated, fail to elicit any of them in their mates (Chapman et al. 1995, Kalb et al. 1993, Tram and Wolfner 1998, Xue and Noll 2000)

Proteins produced by the male's accessory glands (Acps, for ACcessory gland Proteins) have been studied intensively with respect to their roles in the post-mating responses. Over 130 Acps have been identified to date (Chapman 2008, Findlay et al. 2009, Ravi Ram and Wolfner 2007a, Takemori and Yamamoto 2009), and there is an increasing level of understanding concerning their functions. Using a variety of methods, specific Acps have been assigned roles in the induction of ovulation and egg-laying (ovulin, sex-peptide/SP; Aigaki et al. 1991, Chen 1991, Heifetz et al. 2000, Herndon and Wolfner 1995, Peng et al. 2005), induced female refractoriness (SP), sperm competition (Acp62F, Acp29AB; Clark et al. 1995, Fiumera et al. 2005, Fiumera et al. 2007, Mueller et al. 2008, Wong et al. 2008), and maintenance of the post-mating responses over the long-term (SP, CG9997, CG1757, CG1652, CG1656; Peng et al. 2005; Ravi Ram and Wolfner 2007b, Ravi Ram and Wolfner 2009).

The *Drosophila melanogaster* Acp ovulin (Acp26Aa) has been particularly well characterized. Ovulin is a 264 amino acid prohormone that induces ovulation within the first day after it is received by the mated female (Heifetz et al. 2000, Herndon and Wolfner 1995). Like other prohormones, *D. melanogaster* ovulin occurs as a dimer (or other oligomer) (Wong et al. 2006), and is cleaved into several short peptides following mating (these peptides are referred to as CP1, CP2, CP3N, and CP3C) (Heifetz et al. 2005, Park and Wolfner 1995). Two of ovulin's cleavage products, CP3N and CP3C, are sufficient to induce ovulation (Heifetz et al. 2005), consistent with the hypothesis that cleavage releases bioactive peptides. At least two male-derived proteins, the Acps CG11864 (a protease) and Acp62F (a protease inhibitor), regulate cleavage of ovulin (Mueller et al. 2008, Ravi Ram et al. 2006), and female factors are also involved (Park and Wolfner 1995, Sirot et al. unpublished).

Perhaps surprisingly, ovulin's sequence diverges very rapidly between species, despite its important function. Protein divergence between ovulin orthologs from the close relatives *D. melanogaster* and *D. simulans* is about 15%, in contrast to a genome-wide average divergence of 1–2% (Begun et al. 2007). Ovulin's putative ortholog in the more distant relative *D. pseudoobscura* is about 80% divergent (Wagstaff and Begun 2005). This high rate of divergence does not appear to be due to low functional constraint: molecular population genetic data suggest a history of positive selection for amino acid changes within this protein, with an excess of non-synonymous substitutions between species (Aguade 1998, Tsaur and Wu 1997), evidence of a recent sweep in *D. melanogaster* (Fay and Wu 2000), and possible balancing selection in *D. mauritiana* (Tsaur et al. 1998). The mechanisms responsible for selection on ovulin remain unclear, although sperm competition and sexual conflict have been both proposed (Aguadé 1998, Aguadé et al. 1992, Tsaur et al. 1998, Tsaur and Wu 1997, Wong et al. 2006). Acps in general are thought to be the targets of post-copulatory sexual selection due to their various functional roles (e.g., Cameron et al. 2007, Clark et al. 2006). Sperm competition, for example, should favor alleles that promote sperm storage or that allow sperm to resist displacement by sperm from subsequent males. Moreover, conflict between males and females over post-mating processes, e.g., the rate of egg-laying, may drive an evolutionary "arms-race" that leads to rapid seminal protein evolution. A recent study showed that males

transfer more ovulin to their mates when the risk of sperm competition is higher, possibly increasing paternity prior to female remating (Wigby et al. 2009). Structure-function studies of ovulin should help to further elucidate this protein's mode of action, and may shed light on features that contribute to its high rate of evolution.

Despite ovulin's rapid sequence divergence between species, we previously noticed some short conserved sequence motifs within ovulin's C-terminus (Wong et al. 2006). Computational sequence analysis suggested that these motifs might be capable of forming coiled-coils, a common protein-protein interaction motif (Parry et al. 2008). Given that *D. melanogaster* ovulin is capable of dimerization, we hypothesized that these putative coiled-coils are involved in ovulin's dimerization in other *Drosophila* species, as well as in *D. melanogaster*. Here, we test these hypotheses. Using yeast two-hybrid and *in vitro* cross-linking methods, we find that ovulin proteins (and/or their C-termini) from non-*melanogaster* species of *Drosophila* also form dimers despite the overall high levels of amino acid sequence divergence, consistent with the conservation of important dimerization motifs. For cases in which interspecific hybrid *Drosophila* could be generated, we tested whether interspecific dimers of ovulin could form *in vivo*. We found that heterospecific ovulin dimers can form *in vivo* between molecules that are 15% divergent overall, again suggesting a broad conservation of structure. Using a mutagenesis approach, we then showed that conserved residues in one of ovulin's putative coiled-coils, as well as two tyrosine residues N-terminal to this putative coiled-coil, are in fact necessary for the dimerization of ovulin's C-terminal 45 amino acids (the minimal dimerization motif previously identified). Thus, our results show that conserved coiled-coil and tyrosine motifs are necessary for ovulin's dimerization, and that dimerization is itself conserved across substantial sequence divergence. These findings raise the possibility that coiled-coil proteins might generally evolve rapidly, due to the low primary sequence requirements of the coiled-coil structure. To test this hypothesis computationally, we examined the evolutionary rates of over 8500 *Drosophila* proteins, and found that putative coiled-coil proteins tend to evolve more rapidly than proteins predicted to lack coiled-coils, consistent with our prediction.

2. Materials and Methods

2.1. Fly rearing

Strains of *D. melanogaster* (Canton-S) and *D. simulans* (Sim6) were maintained on yeast-glucose media at room temperature on 12 hour light:12 hour dark cycles. *D. melanogaster*/*D. simulans* hybrids were created using the *lethal hybrid rescue (lhr)* gene: viable hybrid male offspring can be produced by mating *D. simulans lhr⁻* males to virgin Canton S *D. melanogaster* females (Barbash et al. 2000).

2.2. Analysis of accessory gland extracts

For analysis of SDS-stable ovulin complexes in pure (non-hybrid) *D. melanogaster* and *D. simulans* males, we followed the protocol of Wong et al. (2006). Briefly, accessory glands were dissected into 50 μ l of 40% sucrose plus protease inhibitor cocktail (Roche) and ground with a pestle. 2xSDS-PAGE loading buffer, with or without 0.1% 2-ME, was then added to this extract. For experiments to detect interaction of ovulins in hybrid *D. melanogaster*/*D. simulans* males and pure-species controls, male accessory glands were dissected directly into the buffer described in Yu et al. (1999) and ground with a pestle. SDS-PAGE loading buffer without 2-ME was then added, and samples were separated by 7.5% SDS-PAGE. Western blotting was performed according to standard protocols, using anti-ovulin antibodies at a 1:2000 dilution and secondary antibodies at the same concentration.

2.3. Cloning, *in vitro* protein synthesis, and protein cross-linking

We chose to focus on the role of sequences within ovulin's C-terminus in self-interaction, since a 45-amino acid C-terminal fragment is sufficient for self-interaction in yeast two-hybrid assays (Wong et al. 2006). We used chemical cross-linking of C-terminal ovulin fragments to investigate the self-interaction of several ovulin mutants, and of ovulin orthologs from different species of *Drosophila*. 3' fragments of the *ovulin* gene were amplified by polymerase chain reaction (PCR) from *D. melanogaster* (aa 219-264 of 264), *D. simulans* (aa 156-255 of 255), *D. yakuba* (aa 179-234 of 234), and *D. pseudoobscura* (aa 209-247 of 247), and cloned in the Gateway compatible entry vector pENTR-dTopo (Invitrogen; Carlsbad, CA) according to the manufacturer's instructions (primer sequences available upon request). Site-directed mutagenesis of the *D. melanogaster* entry clone was performed using the QuikChange kit (Stratagene; Cedar Creek, TX), and mutations were verified by Sanger sequencing at the Cornell Life Sciences Core Laboratories Center. Expression constructs for *in vitro* protein synthesis in the vector pEXP1-DEST (Invitrogen) were generated by LR reaction (Invitrogen).

In vitro protein synthesis was performed using the Expressway Mini Cellfree Expression System (Invitrogen) according to the manufacturer's protocol. Cross-linking was performed by adding 10 μ l of 10mM dimethyl suberimidate•2 HCl (DMS) in phosphate buffered saline pH 8.0 (PBS) to 10 μ l of the *in vitro* protein synthesis mixture and incubating for 1 hour at room temperature. DMS consists of two reactive imidoester groups separated by a 11.0 angstrom spacer arm; the imidoester groups react with amine groups, found primarily on lysine side chains and the N-termini of proteins, to form covalently cross-linked structures. We used DMS (rather than non-reducing conditions; (Wong et al. 2006)), to examine self-interaction of C-terminal ovulin fragments since preliminary data (not shown) suggested that non cross-linked dimers were not SDS-stable, perhaps due to the absence of Cys199 in these peptides. Controls were performed using 10 μ l of PBS without DMS. 20 μ l of SDS-PAGE sample buffer was added to stop the reaction. Proteins were separated by SDS-PAGE, and detected by Western blotting using α -His antibodies (Sigma) at a 1:2500 concentration.

2.4. Yeast two-hybrid analysis

The self-interaction of ovulin orthologs in non-*melanogaster* species, as well interactions between ovulin orthologs from different species, was also investigated using yeast-two hybrid analysis. Ovulin coding sequences, minus those encoding the predicted signal sequence, were amplified by PCR from males of each of 9 *Drosophila* species (*D. melanogaster*, *D. simulans*, *D. sechellia*, *D. mauritiana*, *D. yakuba*, *D. teissieri*, *D. pseudoobscura*, *D. persimilis*, *D. miranda*), and cloned into pENTR-dTOPO using the Gateway system (Invitrogen). The *ovulin* genes were then subcloned into each of two Gateway-compatible yeast two-hybrid vectors (pGAD, pGBKT7) using the LR reaction (original vectors from Clontech, modified by K. Ravi Ram, A. Garfinkel, and M.F.W., unpublished data). Haploid yeast strains AH109 and Y187 (mating type **a** and mating type **alpha**) bearing ovulin constructs were mated in all possible pairwise combinations, and interactions were scored by growth on media lacking histidine and adenine.

2.5. Analysis of rates of substitution

Estimates of the non-synonymous substitution rate dN , and of the normalized ratio of non-synonymous to synonymous substitution rates ω , and inferences of positive selection were performed using PAML (Yang 1997) by Larracuent et al. (2008) for 8510 genes (not including *ovulin*) with clear one-to-one orthologs in 6 species of the genus *Drosophila*: *D. melanogaster*, *D. simulans*, *D. sechellia*, *D. yakuba*, *D. erecta*, and *D. ananassae*. Briefly, gene-averaged dN and ω were estimated under model M0, which assumes a single value of dN and dS for each gene across the entire phylogeny. For inferences of positive selection, model M7 was used as the null hypothesis, allowing beta-distributed variation in ω but disallowing

codons with $\omega > 1$. The alternative hypothesis M8 also allows beta-distributed variation in ω , and adds a class of codons with $\omega > 1$. For each gene, a likelihood ratio test can be used to determine if the data fit model M8 better than they fit model M7; a rejection of M7 constitutes evidence in favor of recurrent positive selection on a subset of codons. False discovery rate (FDR) corrections were performed as described in Larracuente et al. (2008).

We examined the influence of several factors on dN , ω , and the likelihood of positive selection: presence/absence of putative coiled-coil domains, protein length, tissue specificity, maximum expression level, length and number of introns, and local recombination rate. The presence of one or more coiled-coil domains was predicted using Paircoil2 (McDonnell et al. 2006), using default parameters. All other factors are described in Larracuente et al. (2008). We used multiple linear regression in order to infer the contribution of each factor to dN and ω , both of which are continuous variables. Logistic regression was used to infer the contribution of each factor to positive selection, where the outcome for each gene is binary (selected or not selected). Statistical analyses were performed using R version 2.5.1 (R Core Development Team 2009).

3. Results and Discussion

3.1. Ovulin contains conserved potential self-interaction motifs

Previously, we showed that ovulin's 147 aa C-terminal cleavage product (CP3C) is capable of self-interaction (Wong et al. 2006). Moreover, within this cleavage product, the C-terminal 45 amino acids of ovulin are capable of self-interaction in yeast two-hybrid assays (ovulin's C-terminal 45 amino acids will be referred to as "C45" hereafter). These findings prompted us to look for potential interaction motifs in ovulin's C-terminus. We have identified three such motifs, all of which are highly conserved (Fig. 1A), despite the overall high sequence divergence in ovulin across these species: (1) In Wong et al. (2006), we suggested that three potential coiled-coil motifs may be present in CP3C (amino acids 126-136, 171-189, 229-246). Coiled-coils consist of two or more interacting α -helices, each of which has a strongly hydrophobic face. Participating α -helices wrap around each other by virtue of hydrophobic interactions between these surfaces. The hallmark of a coiled-coil, then, is an α -helix with hydrophobic amino acids occurring every 3-4 residues. (2) We also previously suggested that ^{199}Cys may participate in an inter-subunit disulfide bond. Consistent with a role for a disulfide bond in dimerization, putative ovulin dimers are SDS-stable in the absence of reducing agent (Wong et al. 2006). (3) A $^{224}\text{Yxxx}^{228}\text{Y}$ motif may also contribute to self-interaction; this short domain was not previously noted or suggested as a possible ovulin self-interaction motif. A similar YxxxY motif is known to mediate interactions between mammalian thymine DNA glycosylase (TDG) and SRC1 (Lucey et al. 2005); this finding, combined with the conservation of this motif (see below), led us to consider ovulin's $^{224}\text{Yxxx}^{228}\text{Y}$ motif as a potential self-interaction sequence.

The two candidate self-interaction motifs present in C45, $^{224}\text{Yxxx}^{228}\text{Y}$ and one coiled-coil, show remarkable conservation in this otherwise rapidly evolving protein (Fig. 1B). Both tyrosines in the $^{224}\text{Yxxx}^{228}\text{Y}$ motif are conserved in all six species we examined, spanning approximately 25 million years and ~80% amino acid divergence across the ovulin protein. Moreover, although the YxxxY motifs of mammalian TDG and SRC1 do not appear to have a strong requirement for charged residues (Lucey et al. 2005), residues between ovulin's conserved tyrosines tend to carry a charge. In particular, the third intervening residue is charged in all species for which sequences are available. Finally, we also note that ^{199}Cys is conserved between species, although it is not present in C45 (data not shown). The conservation of these putative self-interaction domains suggests that ovulin's dimeric structure may also be conserved, despite extensive amino divergence.

3.2 Self-interaction of putative ovulin orthologs from other species of *Drosophila* suggests a conserved role for interaction motifs

Given the conservation of all three putative self-interaction motifs in ovulin, we predicted that ovulin orthologs from other species of *Drosophila* should also self-interact. Yeast two-hybrid analyses indicated that ovulin orthologs from *D. melanogaster*, *D. simulans*, *D. sechellia*, and *D. mauritiana* are all capable of self-interaction in this system (Fig. 2). *D. simulans*, *D. sechellia*, and *D. mauritiana* are closely related species, and diverged from *D. melanogaster* approximately 5 million years ago; overall ovulin divergence between the *simulans* cluster and *melanogaster* is about 15%. Using yeast two-hybrid analyses, we did not detect self-interaction of ovulin orthologs from the more distantly related species *D. yakuba*, *D. teissieri*, *D. pseudoobscura*, *D. persimilis*, *D. miranda*. However, it is difficult to interpret such negative data, since low protein yield or misfolding can cause negative results, rather than a 'true lack' of interaction.

To verify our yeast two-hybrid results for *D. simulans*, we used Western blotting of accessory gland extracts following non-reducing SDS-PAGE. We prepared accessory gland extracts from *D. melanogaster* and *D. simulans*, and performed SDS-PAGE in the presence or absence of the reducing agent 2-ME (Fig. 3A). As observed previously, *D. melanogaster* ovulin migrates as three bands of 36 kD, 37 kD and 41 kD under reducing conditions, representing different glycosylation forms (Monsma et al. 1990). In the absence of 2-ME, however, it runs at ~80 kD, consistent with a disulfide bonded dimer. We obtained analogous results for the putative ovulin ortholog of *D. simulans* (Fig. 3A), consistent with self-interaction of ovulin in this species. Monomeric ovulin from *D. simulans* migrates at approximately 35 kD, with the putative dimer migrating at 70 kD. Thus, as predicted from our yeast two-hybrid results, *D. simulans* ovulin (as well as *D. melanogaster* ovulin) self-interacts *in vivo*. Although we attempted to measure ovulin self-interaction in extracts from other *Drosophila* species, the high protein divergence of ovulin orthologs from *D. yakuba*, *D. teissieri*, and *D. takahashii* prevented us from using this method to test *in vivo* interactions of those species ovulins: our antibodies, which were raised against *D. melanogaster* ovulin, did not recognize proteins in these other species strongly enough to permit this experiment.

We therefore used an *in vitro* test to determine whether the interactions identified in our yeast two-hybrid screen occurred in ovulin orthologs from several other *Drosophila* species. We predicted that C-terminal fragments of ovulin from non-*melanogaster* species of *Drosophila* should be capable of self-interaction, given the concentration of conserved interaction motifs in ovulin's C-terminus. We expressed 6xHis-tagged C-terminal peptides of ovulin (orthologous to the C-terminal 45 amino acids of *D. melanogaster* ovulin) from *D. simulans*, *D. yakuba*, and *D. pseudoobscura* in cell free *E. coli* extracts and assayed self-interaction by DMS cross-linking (Fig. 3B). Note that, for this experiment, peptides were detected using an antibody against the 6xHis tag, so that sequence divergence between ovulin proteins from different *Drosophila* species did not affect detection sensitivity. As predicted, products whose molecular weight were consistent with dimers were present following cross-linking for all species assayed. Different levels of dimer formation were observed for different species; in particular, the putative dimer signal is relatively weak for *D. yakuba*. This may reflect differences in the strengths of inter-subunit associations; alternatively, different lysine content and positioning for different species could contribute to differences in cross-linking efficiency regardless of bond strengths. Overall, our two-hybrid, non-reducing PAGE, and cross-linking experiments demonstrate that ovulin's dimeric structure is conserved between species, likely reflecting the conservation of its proposed self-interaction motifs.

3.3. Ovulins of different *Drosophila* species can cross-interact

We reasoned that if ovulin's tertiary structure is highly conserved, as suggested by the conservation of putative interaction motifs and dimerization of non-*melanogaster* ovulins, then ovulin orthologs from different species should be able to interact with each other. We investigated this possibility using *D. melanogaster*/*D. simulans* hybrid males, whose accessory glands should produce ovulin from both genomes. *D. simulans* and *D. melanogaster* ovulins are 15% diverged, but the potential interaction motifs noted in section 3.1 are conserved. *D. simulans* ovulin dimers migrate at a higher gel mobility than do *D. melanogaster* ovulin dimers on SDS-PAGE; thus, hybrid dimers should run at an intermediate gel mobility. Western blot analysis of hybrid male accessory glands finds exactly this outcome (Fig. 4), strongly suggesting the presence of heterospecific dimers (in addition to the predicted homospecific dimers). Consistent with this *in vivo* result, yeast-two hybrid analyses suggest that *D. melanogaster* ovulin and its orthologs in *D. simulans*, *D. sechellia*, and *D. mauritiana* are capable of forming heterospecific dimers (Fig. 2). Thus, it appears that the tertiary structure of ovulin is sufficiently conserved to allow dimerization even in the face of 15% amino acid divergence protein-wide.

These results suggest that similar structures are adopted by the putative coiled-coil domains of ovulin orthologs from a variety of species, despite an overall high rate of protein divergence: Dimerization is conserved in the face of up to 80% protein divergence between *D. melanogaster* and *D. pseudoobscura*, and inter-specific dimers can form between orthologs diverged by 15%. Thus, it appears that ovulin's dimeric structure is robust to very high levels of primary sequence divergence, with only a few residues conserved in the putative dimerization domain.

3.4. Conserved coiled-coil and YxxxY motifs are necessary for the self-interaction of C45 in vitro

The dimerization of ovulin orthologs from a range of different species suggested that the conserved motifs in ovulin's C-terminus may indeed be important for self-association. We therefore used a mutagenesis approach to test directly whether the conserved YxxxY and coiled-coil motifs were essential for self-interaction of C45 of *D. melanogaster* ovulin. Roles for ovulin's putative coiled-coils and YxxxY motifs in ovulin self-interaction had previously only been hypothesized (Wong et al. 2006; this study). Wild-type *D. melanogaster* C45 tagged at its N-terminus with 6xHis and Xpress (Invitrogen) epitopes, or mutants bearing alterations of residues with predicted roles in self-interaction (Fig. 5A), were produced using a cell-free *E. coli* extract (Invitrogen). The products were either cross-linked using DMS or incubated in buffer (PBS) alone. Wild-type C45 incubated in buffer alone runs at about 9 kD on an SDS-PAGE gel, consistent with a monomeric peptide; a product of ~18 kD is present when wild-type C45 is cross-linked with DMS (Fig. 5B), consistent with the presence of cross-linked dimers. A smaller product of ~15 kD is also visible following DMS treatment, and may represent a degradation product.

We made three mutants that are predicted to disrupt the coiled-coil: Zip1, Zip2, and Zip3 each bear two alanine mutations (aa's 229 and 232, 236 and 239, 243 and 246, respectively) of residues predicted to lie within the hydrophobic core of the coiled-coil (Fig. 5A). Each of the coiled-coil mutants is predicted to disrupt the α -helix's hydrophobic face through two turns of the helix. Higher molecular weight products were not detectable following DMS treatment for any of these three mutants, suggesting that they are incapable of forming dimers. This result is consistent with a coiled-coil interaction interface between monomers of C45.

Similarly, mutations of the ²²⁴Yxxx²²⁸Y motif greatly reduce or eliminate C45's ability to self-interact. No dimer is detected following DMS treatment when both tyrosines in

the ²²⁴Yxxx²²⁸Y motif are converted to alanines (Fig. 5B). We attempted to assay self-interaction of a double Y->F mutant, but protein yields were too low to draw robust conclusions.

The conservation of residues necessary for self-interaction, as well as the conservation of dimeric structure, strongly suggests that dimerization is necessary for ovulin function. Ideally, we would like to test the functional significance of ovulin dimerization, i.e., experimentally demonstrate that dimerization is necessary for the induction of egg-laying. Technical considerations make this an extremely difficult task, however. First, the presence of multiple domains with potential roles in self-interaction may make it difficult to abolish dimerization completely. The data presented in this paper show that pairs of point mutations eliminate self-interaction of C45, but ovulin's two other putative coiled-coils and a potentially important cysteine residue are located outside of this peptide (Fig. 1). Second, if dimerization mutants were unable to induce ovulation, it would be difficult to attribute the functional deficit to the protein's inability to dimerize *per se* – mutated residues could also be involved in receptor binding, for example. Structural studies of ovulin complexed to its as yet unidentified receptor (s) may instead represent a more promising method for determining the functional significance of dimerization.

3.5. Evidence for reduced constraint on predicted coiled-coil proteins

We suggest that ovulin's coiled-coil structure imposes few constraints on its primary sequence, as shown by the dimerization of ovulin orthologs diverged by up to 80% protein-wide. We propose that the constraints imposed by ovulin's tertiary structure are limited and local to specific domains, such that variation in other parts of the protein does not prevent dimer formation or destabilize the dimer. Additional structural constraints might be imposed by, for example, ligand-receptor interactions, but the requirements for dimerization appear to be minimal. We therefore predicted that other proteins bearing coiled-coil domains should also show reduced constraint, as indicated by an increased rate of amino acid evolution (*dN*), and an increased *dN/dS* ratio (ω).

We have tested this hypothesis using the sequences of the genomes of 6 species of *Drosophila* (Clark et al. 2007, Larracuente et al. 2008), using Larracuente et al.'s (2008) estimated evolutionary rates and inferences of positive selection for 8510 genes with clear one-to-one orthologs in *D. melanogaster* and its five closest sequenced relatives, *D. simulans*, *D. sechellia*, *D. yakuba*, *D. erecta*, and *D. ananassae*. We compared rates of nucleotide divergence between proteins containing predicted coiled-coils, and those without predicted coiled-coils (regardless of the presence or absence of other domains). After controlling for several other parameters previously demonstrated to contribute to variation in the rate of amino acid substitution (tissue specificity, expression level, protein length, intron number and length, recombination rate; Larracuente et al. 2008), we found that predicted coiled-coil proteins tend to have a higher *dN* and ω than do proteins lacking a predicted coiled-coil (Table 1; *n* = 1824 proteins with a predicted coiled-coil, 6685 predicted to lack a coiled-coil; *dN*: *P* = 1.6×10^{-4} ; ω : *P* = 0.0035). This difference in the rate of amino acid evolution does not appear to be due to differences in levels of positive selection, as we find no differences in the proportions of predicted coiled-coil and non-coiled-coil proteins inferred to have experienced positive selection (Table 2; *P* = 0.147). We note that many coiled-coil proteins interact via heterologous coiled-coils (e.g., Vinson et al. 2006); this is not problematic for our analysis, since coiled-coils still fulfill important structural roles, whether in homologous or heterologous interactions. Thus, consistent with our prediction, putative coiled-coil proteins appear to be under less amino acid constraint than do proteins lacking predicted coiled-coils. Further consideration of the impact of structural motifs such as coiled-coils, as well as other sources of constraint, on rates of amino acid substitution, should prove a fruitful avenue for future studies.

In conclusion, we have shown that despite their great overall sequence difference, ovulin proteins from a wide range of species are capable of dimerization, and that this dimerization is mediated at least in part by several short conserved motifs within ovulin's C-terminal region. Only a few residues appear to be required for self-interaction, since dimerization is robust to high levels of overall amino acid divergence. Our comparative analysis suggests that coiled-coil proteins may in general be robust to sequence divergence.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

Conserved putative interaction motifs of ovulin. Panel A: Schematic of *D. melanogaster* ovulin. Three kinds of motif are hypothesized to contribute to self-interaction: Putative coiled-coils (blue), the ²²⁴Yxxx²²⁸Y motif (green), and ¹⁹⁹Cys. The putative secretion signal sequence is indicated in yellow, and putative proteolytic cleavage sites are marked with arrows. The C-terminal 45 amino acids of ovulin (C45) were previously shown to be sufficient for self-interaction in yeast two-hybrid assays (Wong et al. 2006). Panel B: Predicted amino acid sequence of *D. melanogaster* C45 (mel) and orthologous regions from *D. simulans* (sim), *D. teissieri* (tei), *D. yakuba* (yak), *D. pseudoobscura* (pse), and *D. miranda* (mir). This fragment of ovulin contains the ²²⁴Yxxx²²⁸Y motif (green) and one putative coiled-coil (blue). Residues predicted to form the hydrophobic face of the coiled-coil are indicated with asterisks.

	BD	<i>mel</i> -BD	<i>sim</i> -BD	<i>sec</i> -BD	<i>mau</i> -BD	<i>tei</i> -BD	<i>yak</i> -BD	<i>mir</i> -BD	<i>per</i> -BD	<i>pse</i> -BD
AD	-	-	-	-	-	-	-	-	-	-
<i>mel</i> -AD	-	+	+	+	+	-	-	-	-	-
<i>sim</i> -AD	-	+	+	+	+	-	-	-	-	-
<i>sec</i> -AD	-	+	+	+	+	-	-	-	-	-
<i>mau</i> -AD	-	+	+	+	+	-	-	-	-	-
<i>tei</i> -AD	-	-	-	-	-	-	-	-	-	-
<i>yak</i> -AD	-	-	-	-	-	-	+/	-	-	-
<i>mir</i> -AD	-	-	-	-	-	-	-	+/	-	-
<i>per</i> -AD	-	-	-	-	-	-	-	-	-	-
<i>pse</i> -AD	-	-	-	-	-	-	-	-	-	-

+: strong interaction; +/-: weak interaction; -: no detectable interaction. Faint, empty cells: not tested.

Figure 2.

Yeast two-hybrid interactions of ovulin orthologs from 9 *Drosophila* species. Species are abbreviated in the order: *D. melanogaster*, *D. simulans*, *D. sechellia*, *D. mauritiana*, *D. teissieri*, *D. yakuba*, *D. miranda*, *D. persimilis*, *D. pseudoobscura*. “AD” and “BD” are empty activation domain and binding domain vectors. Cells containing “+” showed strong interaction (growth on medium lacking histidine and adenine), cells containing “+/” showed weak interactions (growth on only selective medium lacking histidine), cells containing “-” were tested but showed no detectable interactions. The faint, empty cells denote combinations that were not tested. All boxes tested combinations showed growth on media lacking leucine and tryptophan which were controls for maintenance of both two-hybrid plasmids.

Figure 3.

Putative ovulin complexes in non-*melanogaster* species. Panel A: Western blots using anti-ovulin antibodies on accessory gland extracts from *D. melanogaster* (mel) and *D. simulans* (sim) in the presence (+) or absence (-) of the reducing agent 2-ME. Monomers run at 36–41 kD (*D. melanogaster*) or 35 kD (*D. simulans*), and putative dimers are present at 80 kD and 70 kD, respectively. Panel B: Western blots of *D. melanogaster* C45 and C-terminal ovulin fragments from *D. simulans*, *D. yakuba*, and *D. pseudoobscura* produced *in vitro*, following treatment with buffer alone (-) or with DMS (+). Expected monomeric molecular weights are 9 kD, 13 kD, 11 kD, and 7 kD, respectively. Note the presence of a product consistent with a

dimer in all species following treatment with DMS. Extra bands below 13 kD (*D. simulans*) or 11 kD (*D. pseudoobscura*) may be degradation products.

Figure 4.

Putative heterospecific ovulin dimer in *D. melanogaster*/*D. simulans* hybrids. Accessory gland extracts from *D. melanogaster* (mel), *D. simulans* (sim), or hybrid males were separated under non-reducing conditions. Two bands of ~74 and 80 kD are visible in *D. melanogaster*, corresponding to dimers of monomeric 36/37 kD and 41 kD ovulin. In *D. simulans*, a single band of 70 kD represents the dimer. In the hybrid, both *D. melanogaster* bands are visible, as well as a band of ~71–72 kD, the expected size of a heterospecific dimer. Pure *D. simulans* ovulin is not visible in the hybrid lane because of loading differences (more males are represented in the *D. simulans* lane than in the other two).

Figure 5.

Conserved motifs in ovulin C45 are necessary for dimerization. Panel A: Amino acid sequence of *D. melanogaster* C45 and of four mutants assayed for self-interaction. Residues predicted to form the hydrophobic face of the coiled-coil are indicated with asterisks. Mutated residues are highlighted in red. B: Western blots of wild-type and mutant forms of C45 produced *in vitro*, after exposure to the cross-linker DMS (+) or buffer only (-). Wild-type C45 forms SDS-stable products in the presence of DMS, the molecular weight of which is consistent with a dimer (a smaller product at ~16 kD may be a degradation product). Putative coiled-coil mutants (Zip1, Zip2, Zip3) and a mutant with Y->A mutations in the ²²⁴Yxxx²²⁸Y motif (YYAA) fail to form dimers in the presence of DMS. Lanes shown in this figure are derived from two separate experiments (WT, Zip1, and Zip2 are from one experiment, and Zip3 and YYAA are from another experiment).

Table 1Multiple regression of genome-wide contributors to dN and ω .

Parameter ^a	dN		ω	
	β	<i>P</i> -value	β	<i>P</i> -value
Coiled-coil present	0.048	1.6×10^{-4}	0.036	0.0035
Log10(protein length)	0.342	$<2 \times 10^{-16}$	0.217	$<2 \times 10^{-16}$
Specificity	0.473	$<2 \times 10^{-16}$	0.482	$<2 \times 10^{-16}$
Log10(max. expression)	-0.047	1.6×10^{-7}	-0.031	2.3×10^{-4}
Log10(# introns + 1)	-0.495	$<2 \times 10^{-16}$	-0.280	$<2 \times 10^{-16}$
Intron length	-7.5×10^{-6}	1.3×10^{-8}	-2.4×10^{-6}	0.056
Recombination rate	-0.013	0.0011	-0.01	0.0072

^aFactors contributing significantly to either dN or ω were chosen according to Larracuente et al. (2008), and presence/absence of a coiled-coil was predicted using Paircoil2 (McDonnell et al. 2006). 1824 genes encoding predicted coiled-coil proteins and 6685 encoding predicted non-coiled-coil proteins were used for this analysis.

Table 2

Logistic regression of genome-wide contributors to positive selection.

Parameter	β	P-value
Coiled-coil present	0.129	0.147
Log10(protein length)	1.41	$<2 \times 10^{-16}$
Specificity	0.769	$<9.8 \times 10^{-11}$
Log10(max. expression)	0.151	0.021
Log10(# introns + 1)	-0.384	0.009
Intron length	-6.3×10^{-6}	0.528
Recombination rate	-0.013	0.772